Thiamine antimetabolite causes functional thiamine deficiency in patients with end stage renal disease

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Decreased transketolase activity is an unexplained characteristic of patients with end stage renal disease (ESRD) and is linked to impaired metabolic and immune function. Herein we describe the discovery of a link to impaired functional activity of thiamine pyrophosphate co-factor through the presence, accumulation and pyrophosphorylation of the thiamine antimetabolite, oxythiamine, in renal failure. Plasma oxythiamine was increased 4-fold in patients receiving continuous ambulatory peritoneal dialysis (CAPD) and 15-fold in patients receiving haemodialysis (HD) immediately before a dialysis session: healthy controls 0.18 (0.11 – 0.22) nM, CAPD, 0.64 (0.48-0.94) nM and HD (2.73 (1.52-5.76) nM); P<0.001, *Mann-Whitney U test*. Oxythiamine was converted to the transketolase inhibitor oxythiamine pyrophosphate (OTPP). Red blood cell OTPP concentration was increased 4-fold in HD: healthy controls, 15.9 ± 10.4 nM and HD patients, 66.1 ± 26.7 nM; P<0.001, *t-test*. This accounted for the concomitant 41% loss of transketolase activity (mU/mg Hb): healthy controls, 0.410 ± 0.144 and HD, 0.240 ± 0.107; P<0.01, *paired t-test*. This may be corrected by displacement with excess thiamine pyrophosphate and explain lifting of decreased transketolase activity by high dose thiamine supplementation in previous studies. Oxythiamine is likely of dietary origin, through cooking of acidic thiamine-containing foods. Trace level oxythiamine was not formed from thiamine degradation under physiological conditions but rather under acidic conditions at 100 ^{o}C. Monitoring of plasma oxythiamine concentration in renal failure and implementation of high dose thiamine supplements to counter it may help improve clinical outcome of patients with renal failure.

Loss of clearance in chronic kidney disease (CKD) leads to accumulation of waste products from metabolism that increase to potentially damaging concentrations and thereby become uremic toxins. In end stage renal disease (ESRD) potentially noxious metabolites may increase >10-fold, particularly preceding a dialysis session. Among classes of uremic toxins are catabolic and degradation products of essential nutrients and cofactors. Where similar in structure to their precursor but non-functional, uremic toxins may have potentially damaging function as anti-metabolites.[1]

It is known that there is impaired function of the pentosephosphate pathway in uraemia at the thiamine pyrophosphate (TPP)-dependent step catalysed by transketolase[2]. The inhibition of transketolase was reversible although the identity of the inhibitor was difficult to discern. The inhibitor was of low molecular weight and initially considered to be guanidinosuccinic acid (GSA). Low levels of GSA in plasma of patients with decreased red blood cell transketolase activity, lack of correlation of GSA concentration to inhibition of transketolase activity and failure of GSA to inhibit transketolase activity in red blood cells *ex vivo* suggested other compound(s) are likely involved.[3-5] Disturbance of levels of pentosephosphate pathway metabolites in peripheral nerves *in vivo* regulated by transketolase activity and recovery of this by HD indicated reversible inhibition of transketolase. Transketolase activity was also decreased in patients with continuous ambulatory peritoneal dialysis (CAPD).[6] This occurred in the presence of normal levels of plasma thiamine and red blood cell TPP.[4, 7, 8] The mechanism of reversible inhibition of transketolase in renal failure has remained unresolved for over 40 years.

We hypothesised that transketolase may be inhibited in renal failure by an anti-metabolite of thiamine which is normally cleared but accumulates to inhibitory levels with loss of clearance in ESRD. Oxythiamine (4-hydroxythiamine) [9] is pyrophosphorylated by thiamine pyrophosphokinase to oxythiamine pyrophosphate (OTPP) which inhibits TPP-

dependent enzymes[10-12] – Figure 1. Injection of oxythiamine into rats decreased tissue transketolase activity.[13] We report here the accumulation of oxythiamine in plasma and related OTPP in red blood cells of patients with ESRD that likely explains the inhibition of transketolase in renal failure.

RESULTS

We measured plasma thiamine concentration of healthy subjects and patients with ESRD receiving renal replacement therapy - continuous ambulatory peritoneal dialysis (CAPD) and haemodialysis (HD) by the conventional thiochrome assay[14] – Table 1. The plasma concentration of thiamine was within the normal range and even increased in CAPD and HD patients. Oxythiamine is not detectable by the thiochrome assay as it lacks the 4-aminopyrimidinyl moiety required for thiochrome formation.[15] We therefore developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for oxythiamine and OTPP – Figure 2, a. - d. Oxythiamine were analysed in plasma of ESRD patients receiving CAPD or HD. Oxythiamine was detectable at trace levels in plasma of healthy people: median (lower – upper quartile), 0.18 (0.11 – 0.22) nM. In ESRD patients receiving CAPD and HD, however, this was increased 4-fold, 0.64 (0.48-0.94) nM, and 15-fold, 2.73 (1.52-5.76) nM, respectively; $P<0.001$, *Mann-Whitney U test* – Table 1. In a further group of HD patients we measured plasma oxythiamine before and after a 4 h dialysis session and found that oxythiamine decreased by 53 (34 - 64)% during the dialysis session ($n = 12$, $P<0.002$, *Wilcoxon Signed Ranks test*).

We explored the metabolism of oxythiamine and its effect on thiamine metabolism in a subgroup of healthy control subjects and HD patients. We measured the concentration of TPP in red blood cells of healthy subjects and HD patients and found no significant difference. However, plasma oxythiamine was increased 19-fold in these patients and

corresponding OTPP concentration in red blood cells was increased 4-fold – Figure 3, a. - c. OTPP is a reversible competitive inhibitor of transketolase. We measured the transketolase activity of the red blood cells by the conventional test with lysates prepared *ex vivo*. Red blood cell transketolase activity was decreased 41% in HD patients, with respect to healthy controls – Figure 3d. It was also measured in the presence of excess TPP which is used to assess the percentage unsaturation of transketolase or "thiamine effect" but will also increase transketolase activity by displacing competitive inhibitors. With added exogenous TPP there was no increase in transketolase activity of healthy controls (0.436 ± 0.139 versus 0.410 ± 0.144 mU per mg Hb; $P>0.05$, *paired t-test*) but there was a highly significant increase in HD patients (0.332 ± 0.103 versus 0.240 ± 0.107 mU per mg Hb; $P<0.001$, *paired t-test*). This increase in transketolase activity reflects lifting of reversible competitive inhibition and the competitive inhibitor TPP is presence in the red blood cell lysate. There was no correlation of plasma oxythiamine and red blood cell transketolase activity. However, there were highly significant correlations of red blood cell transketolase activity and OTPP concentration ($r = -0.55$, $P<0.01$; *Spearman*) and red blood cell OTPP concentration with plasma oxythiamine concentration ($r = 0.79$, $P<0.001$; *Spearman*).

To deduce if the accumulation of OTPP in red blood cells likely causes significant inhibition of transketolase clinically, the effect of competitive inhibition of OTPP on transketolase activity was deduced from enzyme kinetics considerations - Figure 3e. Comparing the decrease in red blood cell activity of transketolase in HD patients from healthy controls determined experimentally (Figure 3d) with the computed decrease in residual transketolase activity by inhibition with OTPP (Figure 3e), it can be seen that inhibition of transketolase by OTPP accounts for most of the observed decrease of transketolase activity of red blood cells in HD patients. Experimental transketolase activity is

decreased 41% and the computed decrease of fractional residual activity of transketolase is 35% in HD patients - Figures 3d and 3e.

For further demonstration that transketolase activity in red blood cells of HD patients is decreased reversibly by OTPP, we sought to recover transketolase activity by incubation of red blood cells *ex vivo* in short term culture with exogenous thiamine. A relatively high concentration of thiamine (50 μM) was used to drive rapid phosphorylation to TPP and displacement of OTPP from transketolase. With thiamine supplementation, red blood cell transketolase activity was increased to 0.53 ± 0.11 mU/mg Hb (n = 12), similar to that of healthy controls. In the same incubations, the terminal pentosephosphate pathway metabolite ribose-5-phosphate (R5P) was quantified with and without 50 μM thiamine. Supplementation with thiamine increased the red blood cell concentration of R5P with respect to unsupplemented controls to levels similar to those of healthy control subjects[16]: 34.5 ± 14.5 versus 19.1 ± 10.3 nmol/ml packed red blood cells (n = 12; $P<0.01$, *paired t-test*).

A possible source of oxythiamine detected clinically is formation by high temperature processing of thiamine-containing foods under acidic conditions – similar to but not as severe as conditions of oxythiamine synthesis.[15, 17] To model this we incubated 1 μM thiamine in water, pH 7.0, at 37 ^{o}C and 100 ^{o}C, and in 100 mM acetic acid, pH 2.9, at 37 ^{o}C and 100 ^{o}C for 1 h. Oxythiamine was detected only in thiamine solution heated at 100 ^{o}C and pH 2.9. The concentration of oxythiamine formed was 0.56 ± 0.06 nM or 0.06% of thiamine. Similar conversion of dietary thiamine in cooking or commercial food processing with limited clearance over 2 – 3 days could explain the accumulation of oxythiamine to low nanomolar levels in ESRD patients.

DISCUSSION

From the studies herein accumulation and metabolism of the thiamine antimetabolite oxythiamine in renal failure is a major contributory factor to inhibition of transketolase activity and block of the pentosephosphate pathway in HD patients. It likely produces similar effects in renal failure patients with CAPD and pre-dialysis patients. Surveillance of OTPP levels in red blood cells or oxythiamine levels in plasma may be of benefit. Plasma oxythiamine concentration correlated strongly and positively with red blood cell OTPP which, in turn, correlated strongly and negatively with red blood cell transketolase activity. The lack of correlation of plasma oxythiamine with transketolase activity is likely due to clearance of oxythiamine in dialysis sessions whereas OTPP and transketolase are both retained in red blood cells.

The source of oxythiamine in clinical samples is uncertain. Analytical reagent grade thiamine did not contain detectable oxythiamine impurity. Trace mono-oxygenase metabolism of thiamine to oxythiamine by human tissues or intestinal bacteria is possible but unknown; moreover, mono-oxygenases (cytochrome P450 enzymes) tend to be down-regulated in renal failure.[18] The precedent from chemical synthesis and the dependence of kinetics and product distribution of thiamine degradation on pH and temperature suggest processing of thiamine under acidic conditions at high temperatures forms oxythiamine.[15] Model studies herein support this: oxythiamine was only formed in acidic solution at pH 2.9 heated at 100 ^{o}C. pH 2.9 is similar to the acidity of some fruits, diluted vinegar[19] and mean pH of the stomach in the prandial/postprandial period.[20] There is, therefore, likely no significant formation of oxythiamine in the acidic environment of the stomach. Exposure to oxythiamine is rather likely of dietary origin, formed in thiamine-containing foodstuffs during home or commercial cooking. The degradation of thiamine in foodstuffs has mostly been studied for the rapid degradation at high pH which does not form oxythiamine.[21] Examples of thiamine containing foodstuffs with natural low pH are fruits and fruit juices;

canned fruits are heated during commercial processing. Foods may also be made acidic by vinegar, lemon juice and other acidic culinary additives.[19] The use of vinegar in cooking has been proposed as beneficial in the diet of ESRD patients to decrease potassium and magnesium content of vegetables[22] and to decrease formation of advanced glycation endproducts (AGEs) in foods.[23] This requires reappraisal in light of the current studies. Low protein diets for renal patients will affect the exposure to oxythiamine if they concurrently decrease consumption of cooked thiamine-containing acidic foods. The accumulation of oxythiamine in ESRD patients is due to decreased clearance and elimination by dialysis.

Given the requirement for acidic, high temperature processing for oxythiamine formation, high dose thiamine administered as a pharmaceutical at ambient temperature does not lead to oxythiamine formation. Supporting evidence for this is experimental and clinical studies in renal failure with high dose thiamine or related derivatives where transketolase activity was increased. [24-26] Maintained low or further decreased transketolase activity would be expected if formation of oxythiamine had increased.

In preclinical studies administration of oxythiamine decreased the retention of thiamine in tissues[27] and increased the rate of TPP dephosphorylation.[28] Oxythiamine was a weak inhibitor of thiamine transporters[29] and potentiated the toxicity of methylglyoxal[30] which is also elevated in renal failure and precursor of AGEs.[31] Oxythiamine was taken up and phosphorylated by all tissues of rats except brain; the formation of OTPP was inversely proportional to TPP content.[32] Correspondingly transketolase activity was inhibited in all rat tissues by oxythiamine administration except the brain,[13] likely through formation of OTPP. OTPP inhibits other TPP-dependent enzymes with lower potency: for mammalian pyruvate dehydrogenase, the $K_i \approx 70$ nM[12] and 2-oxoglutarate dehydrogenase complex was little affected.[33]

In clinical studies, decreased transketolase activity leads to impairment of oxidative and reductive pentose pathway activity, contributing to impaired phagocyte function for resistance to infection[34] and impaired disposal of triosephosphates.[35] The latter is associated with stimulation of mitochondrial dysfunction and formation of oxidative stress, increased diacylglycerol formation and activation of protein kinase C, activation of the hexosamine pathway and increased formation of methylglyoxal and AGEs.[36] Further disturbance of thiamine metabolism in renal failure occurs by decreased expression of thiamine transporters in small intestine, heart, liver and brain and likely decreased availability of thiamine at these sites.[37] Herein we showed that incubation of red blood cells from HD patients *ex vivo* with thiamine restored normal levels of transketolase activity and R5P concentration, consistent with correction of impaired pentose pathway activity.

Plasma oxythiamine was decreased after a dialysis session in HD patients and hence is cleared by dialysis. Thiamine binds to human serum albumin with a dissociation constants K_D of *ca.* 1 μM.[38] Plasma thiamine measured in the thiochrome assay is the sum of the free and protein-bound forms. Given the high plasma albumin concentration, 40 mg/ml or *ca.* 600 μM, most of thiamine is protein-bound in plasma. The affinity of oxythiamine for albumin is unknown but oxythiamine had similar urinary excretion characteristics to thiamine in rats and therefore is likely also bound to albumin.[27] This will limit the clearance of oxythiamine in dialysis.

Previous studies have assessed transketolase deficiency in ESRD by measuring erythrocyte transketolase activity stimulating index (αETKA). This measures the fractional or percentage increase in activity of transketolase on addition of excess cofactor TPP.[39] We used addition of 348 μM herein - *ca.* 500 fold higher than present physiologically - to provide for effective and rapid displacement of the OTPP competitive inhibitor. Values of αETKA > 1 are conventionally assumed to be due to decreased availability of TPP and presence of apo-

transketolase. In the presence of a competitive inhibitor such as OTPP, however, αETKA also reports on inhibition of transketolase where the 500-fold excess TPP displaces the competitive inhibitor. Consistent with this, herein addition of 500-fold excess TPP increased transketolase activity in HD patients but not in healthy controls. For αETKA to capture reversible inhibition of transketolase, the activity of transketolase is measured immediately on dilution of red blood cell haemolysate before the inhibitor dissociates from the holoenzyme by dilution; and when 500-fold excess TPP is added to the haemolysate, 30 min pre-incubation is performed in the absence of substrate prior to the transketolase activity measurement to allow for displacement of the inhibitor. When transketolase is in the presence of substrate, exchange of TPP cofactor and OTPP inhibitor is very slow.[40] The absolute level of transketolase activity relates to metabolic function in the pentosephosphate pathway whereas αETKA captures the competitive inhibition of transketolase in ESRD patients and unsaturation of transketolase by TPP in thiamine deficiency.

Dynamics of TPP cofactor binding to apo-transketolase indicate that if highly diluted red blood cell lysates are left without substrate at ambient temperature for >30 min prior to assay, OTPP would be released from holo-transketolase and competitive inhibition lost.[40] This may have contributed to discordant reports of decreased transketolase activity in ESRD patients. Studies by Lonergan and co-workers[2, 3] and Kopple *et al*.[41] used a modified method of transketolase activity assessment developed by Dreyfus[42] which was subsequently found to lack specificity and reproducibility[43-45] and is no longer used. Inhibition of transketolase would have been lost if high dilution of red blood cell lysate was prepared without immediate assay of transketolase activity such that OTPP dissociated from the holoenzyme.

In clinical translation, dietary deficiency of thiamine - leading to Beriberi and Wernicke–Korsakoff syndrome if untreated, may be conveniently diagnosed by urinary excretion of thiamine where excretion of ≤0.20 μmol/24 h reflects thiamine nutritional

deficiency.[46] In ESRD patients, plasma thiamine measured by the thiochrome assay is an expected surrogate measure of this. Recent studies in diabetes and CKD suggest there are also abnormalities of thiamine transport due to tissue-specific down regulation of thiamine transporters.[14, 37, 47] Measurement of renal clearance of thiamine is a sensitive clinical marker of this.[14] Red blood cell transketolase activity is valuable to assess impact on the pentosephosphate pathway and αETKA to explore cause of transketolase activity deficit.[48] Where competitive inhibition of transketolase by OTPP or similar antimetabolite is suspected, care is required to ensure the inhibitor does not dissociate from the holoenzyme before the activity is recorded. Plasma oxythiamine offers an assessment of oxythiamine exposure with analytical standard available commercially.

In summary, transketolase activity is likely impaired in red blood cells in ESRD patients by inhibition by the antimetabolite OTPP. The recommendation for patients with stage 3 – 5 CKD to take a supplement of the daily reference intake (DRI)[49] of 1.1 – 1.3 mg thiamine may be insufficient given the increased concentration of OTPP in renal failure. The remedy to the antimetabolite effects of oxythiamine accumulation is pharmaceutical doses of thiamine to produce increased TPP for OTPP displacement from transketolase. Thiamine (30 - 45 mg per day equivalent) in HD patients was studied and found to alleviate transketolase deficiency.[24] These remedial effects found were consistent with reversal of OTPP antimetabolites effects but oxythiamine was not determined and the minimum dose of thiamine required for benefit is uncertain. High dose thiamine supplementation in renal failure is deserving of further attention to assess if it can consistently alleviate the metabolic impairment linked to transketolase insufficiency.

METHODS

Materials

Thiamine, TPP (≥ 95%), oxythiamine chloride hydrochloride (HPLC grade, ≥ 95%) and D-ribose-5-phosphate, disodium salt dihydrate (≥99%) were purchased from Sigma-Aldrich (Poole, U.K.). [4,5,5–*Methyl*-$^{13}C_3$] thiamine chloride (99 atom %) was purchased from Cambridge Isotope Laboratories (Andover, USA). OTPP was prepared from oxythiamine and purified by HPLC as described.[50]

Healthy subject and patients

Peripheral venous blood samples were collected from healthy human subjects and patients with stage 5 CKD receiving CAPD or HD renal replacement therapy. For HD patients, blood samples were collected immediately before and after a 4 h dialysis session. Samples were collected using EDTA as anticoagulant. Blood cells were sedimented by centrifugation (2000g, 10 min) and plasma removed. White blood cell buffy coast was removed and red blood cells retained. Clinical characteristics of the participants are given - Table 1. Healthy human subjects and patients were recruited at the University Hospitals Coventry & Warwickshire NHS Trust, Coventry, U.K., after written informed consent. The study was approved by National Research Ethics Service Committee West Midlands - Coventry & Warwickshire, (REC 05/Q202/26), and Black Country Research Ethics Committee (REC:09/H1202/113). Plasma and red blood cells were stored at -80^{o}C until analysis.

Red blood cell incubation *ex vivo*

Red blood cells from HD patients were washed three times with 4 volumes of phosphate-buffered saline, and with a final wash of 4 volumes of Krebs-Ringer phosphate buffer (120 mM NaCl, 4.8 mM KC1, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 16.5 mM NaH_2PO_4/ Na_2HPO_4, pH 7.4, and 5 mM D-glucose). Red blood cell suspensions (50% v/v; 0.25 ml) were then

incubated for 2 h at 37 ^{o}C in a shaking water bath with and without 50 μM thiamine, as described.[16] Cells were then either collected by centrifugation (2000g, 10 min, room temperature) and transketolase activity assayed or de-proteinised by addition of ice-cold perchloric acid (250 μl, 0.6 M) and analysed for R5P – see below.

Assay of thiamine and TPP by the HPLC thiochrome method.

Plasma thiamine and red blood cell TPP were determined by HPLC with fluorimetric detection as previously described.[14] Briefly, plasma was used without further processing. Red blood cells were washed 4 times with isotonic saline, lysed with 4 volumes of water and membranes sedimented by centrifugation (6000, 10 min, 4 ^{o}C). The supernatant hemolysate was retained. Plasma diluted 5-fold in water (50 μl) or hemolysate (25 μl) was mixed with 20% (w/v) trichloroacetic acid (TCA) for de-proteinisation; and internal standard, chloroethylthiamine (1 μM, 10 μl), added and mixed again. Samples were kept on ice for 10 min and then precipitate was sedimented by centrifugation (6000g, 4 ºC, 10 min). The supernatant was removed and adjusted to pH 4.5 by addition of 2 M sodium acetate (7 μl for plasma,10 μl for haemolysate) and spin-filtered (0.2 μm, 4000 g, 4 ºC, 10 min). The filtrate (40 μl) was analysed by HPLC. Chromatographic conditions used were optimized for thiochrome recovery. Column: 3 x 150 mm C18 column with 3.5 μm particle size and 3 x 20 mm guard column (Xbridge; Waters, U.K.). Mobile phases: A – 10 mM K_2HPO_4/ KH_2PO_4 in water at pH 8.4; B – 10 mM K_2HPO_4/ KH_2PO_4 in 50% methanol at pH 8.4; and C – 30% isopropanol in water with 0.1% trifluoroacetic acid. The flow rate was 0.5 ml/min. The elution profile was: 0 min, 95% A + 5% B; 0 – 20 min, a linear gradient of 5 – 100% B; 20 – 32 min 100% C (column washing); and 32 – 48.5 min 95% A + 5% B (re-equilibration). Samples were derivatised with NaOH-$K_3[Fe(CN)_6]$ solution prepared and mixed with samples immediately prior to injection. The autosampler was programmed to mix 40 μl 15%

NaOH and 10 µl 1% $K_3[Fe(CN)_6]$, then add 10 µl of this to 40 µl of the sample, and finally 25 µl of the derivatised sample injected onto the column for each analysis. Thiochromes formed by derivatisation were detected by fluorescence spectrophotometer at excitation 365 nm and emission 439 nm. The retention times, limits of detection, interbatch coefficient of variation and recoveries were: thiamine 13.1 min, 36 fmol, 1.1% and 97%; and TPP 4.5 min, 51 fmol, 2.9% and 94%. Stock solutions of thiamine and TPP were calibrated by spectrophotometry assuming extinction coefficients of $\varepsilon_{233} = 14.2$ and $\varepsilon_{247} = 13.0$ $mM^{-1}cm^{-1}$, respectively.[51] Oxythiamine is not detectable by the thiochrome assay as it lacks the 4-aminopyrimidinyl moiety required for thiochrome formation.[15]

Assay of red blood cell transketolase activity and ribose-5-phosphate concentration

Red blood cell transketolase activity was determined by the method of Chamberlin *et al*.[52] To determine the thiamine effect, transketolase activity was measured with and without addition of 348 µM TPP. Haemolysate was incubated with and without TPP for 30 min in the absence of substrate prior to the transketolase activity measurement to allow for displacement of the putative OTPP inhibitor by TPP.

Red blood cell R5P concentration was determined by negative ion LC-MS/MS, as we previously described,[53] confirming quantitation by standard addition analysis of authentic analyte.

Assay of oxythiamine and red blood cell OTPP were determined by liquid chromatography-tandem mass spectrometry

Plasma oxythiamine and red blood cell OTPP were determined by LC-MS/MS, operating in positive ion, multiple reaction monitoring (MRM) mode. Chromatographic and mass spectrometric detection conditions are given in Table 2. Detection were normalised to [4,5,5–

methyl-$^{13}C_3$] thiamine internal standard and analyte amounts deduced from calibration curves constructed by assay of 0.01 – 2.0 pmol oxythiamine and 0.1 – 2 pmol OTPP. For plasma oxythiamine, plasma (50 µl) was spiked with 2 pmol of [$^{13}C_3$]thiamine and deproteinised by addition of 20 µl 20% trichloroacetic acid (TCA). The precipitate was centrifuged (20,000g, 4ºC, 10 min) and the supernatant removed, filtered (0.2 µm pore size) and an aliquot (45 µl analysed by LC-MS/MS. For red blood cell OTPP, packed red blood cells (50 µl) were spiked with 0.1 pmol of [$^{13}C_3$]thiamine (5 µl, 0.02µM) and lysed and de-proteinized by addition of 40 µl 10% TCA. The samples were placed on ice for 10 min and then membranes and protein precipitate sedimented by centrifugation (20,000g, 10 min at 4°C). The supernatant was removed filtered (0.2 µm pore size) and an aliquot (40 µl) analysed by LC-MS/MS. There was no degradation of thiamine to oxythiamine in pre-analytic processing but thiamine degraded partly to oxythiamine in the electrospray source in mass spectrometric detection and so thiamine and oxythiamine were resolved chromatographically to avoid interference – see Figure 2, a. and b. In the assay of OTPP, TPP and OTPP were only partly resolved chromatographically and TPP partly degrades to OTPP in the electrospray sourced of the mass spectrometry. The fraction of TPP degrading to OTPP was constant in analysis of standards and this was used to correct OTPP detected in samples for the contribution produced from TPP degradation to OTPP during electrospray ionization.

Calculation of fractional residual activity of transketolase in the presence of oxythiamine pyrophosphate

The dependence of apparent reaction velocity, V_{app}, of the transketolase-catalysed reaction in the presence of a fixed, steady-state concentration of transketolase and substrates and varied TPP concentration is given by the equation: $V_{app} = k_{app}[TPP]/(K_{TPP} + [TPP])$, where k_{app} is the apparent pseudo first order rate constant, K_{TPP} is the dissociation constant for the

transketolase /TPP complex and [TPP] is the steady-state concentration of TPP.[54] In the presence of the competitive inhibitor OTPP, this is modified to $V_{app,OTPP} = k_{app}[TPP]/(K_{TPP}(1+[OTPP]/K_{OTPP}) + [TPP])$, where K_{OTPP} is the dissociation constant for the transketolase /OTPP complex and [OTPP] is the steady-state concentration of OTPP. The fractional residual activity of transketolase in the presence of a given concentration of OTPP – the proportion of the total transketolase remaining – is given by $V_{app,OTPP}/ V_{app} = (K_{TPP} + [TPP])/(K_{TPP}(1+[OTPP]/K_{OTPP}) + [TPP])$. The fractional activity of transketolase may be deduced for each subject and HD patient by estimation of [TPP] and [OTPP] and assuming values of K_{TPP} and K_{OTPP}: TPP = 610 nM and $K_{OTPP} \approx 15$ nM.[10, 11]

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DISCLOSURE

All authors declared no competing interests.

REFERENCES

1. Duranton F, Cohen G, De Smet R, *et al.* Normal and Pathologic Concentrations of Uremic Toxins. *J Amer Soc Nephrol* 2012; **23:** 1258-1270.
2. Lonergan ET, Semar M, Lange K. Transketolase Activity in Uremia. *Archi Intern Med* 1970; **126:** 851-854.
3. Lonergan ET, Semar M, Sterzel RB, *et al.* Erythrocyte Transketolase Activity in Dialyzed Patients. *New Engl J Med* 1971; **284:** 1399-1403.
4. Pietrzak I, Baczyk K. Erythrocyte transketolase activity and guandino compounds in hemodialysis patients. *Kidney Internat* 2001; **59:** S97-S101.
5. Kopczyński Z, Dryl-Rydzyńska T. Studies on the effect of low-molecule uremic toxins on the activity of glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) and transketolase (E.C.2.2.1.1) in human red blood cells. *Acta Physiol Pol* 1988; **39:** 269-275.
6. Pietrzak I, Baczyk K, Ksiazek P. Thiamine blood level and erythrocyte transketolase activity in continuous ambulatory peritoneal dialysis and intermittent peritoneal dialysis patients. *Ann Universitatis Mariae Curie-Sklodowska Medicina* 1995; **50:** 197-203.
7. Sterzel RB, Semar M, Lonergan ET, *et al.* Relationship of nervous tissue transketolase to the neuropathy in chronic uremia. *J Clin Invest* 1971; **50:** 2295-2304.
8. Niwa T, Ito T, Matsui E, *et al.* Plasma level and transfer capacity of thiamin in patients undergoing long-term hemodialysis. *Amer J Clin Nutrit* 1975; **28:** 1105-1109.
9. Soodak M, Cerecedo LR. Studies on Oxythiamine. *J Amer Chem Soc* 1944; **66:** 1988-1989.
10. Gorbach ZV, Kubyshin VL, Maglysh SS, *et al.* Coenzyme Metabolism in Rat-Liver Transketolase. *Biochemistry-Moscow* 1986; **51:** 935-940.

11. Singleton CK, Pekovich SR, McCool BA, *et al.* The Thiamine-Dependent Hysteretic Behavior of Human Transketolase: Implications for Thiamine Deficiency. *J Nutrit* 1995; **125:** 189-194.
12. Strumilo SA, Senkevich SB, Vinogradov VV. Effect of Oxythiamine on Adrenal Thiamine Pyrophosphate-Dependent Enzyme-Activities. *Biomed Biochim Acta* 1984; **43:** 159-163.
13. Brin M. Effects of Thiamine Deficiency and of Oxythiamine on Rat Tissue Transketolase. *J Nutrit* 1962; **78:** 179-183.
14. Thornalley PJ, Babaei-Jadidi R, Al Ali H, *et al.* High prevalence of low plasma thiamine concentration in diabetes linked to marker of vascular disease. *Diabetologia* 2007; **50:** 2164-2170.
15. Windheuser JJ, Higuchi T. Kinetics of thiamine hydrolysis. *J Pharmaceut Sci* 1962; **51:** 354-364.
16. Thornalley PJ, Jahan I, Ng R. Suppression of the accumulation of triosephosphates and increased formation of methylglyoxal in human red blood cells during hyperglycaemia by thiamine in vitro *J Biochem* 2001; **129:** 543-549.
17. Rydon HN. Note on an improved method for the preparation of oxythiamine. *Biochem J* 1951; **48:** 383-384.
18. Dreisbach AW, Lertora JJL. The Effect of Chronic Renal Failure on Drug Metabolism and Transport. *Expert opinion on drug metabolism & toxicology* 2008; **4:** 1065-1074.
19. Bridges MA, Mattice MR. Over two thousand estimations of the ph of representative foods. *Amer J Digestive Dis* 1939; **6:** 440-449.
20. Russell TL, Berardi RR, Barnett JL, *et al.* Upper gastrointestinal pH in 79 healthy, elderly, North-American men and women. *Pharmaceut Res* 1993; **10:** 187-196.

21. Dwivedi BK, Arnold RG. Chemistry of Thiamine Degradation in Food Products and Model Systems - Review. *J Agric Food Chem* 1973; **21:** 54-60.
22. Naito H. Estimation of therapeutic diet using vinegar for renal failure patients changes in elution rate of potassium sodium calcium and magnesium in fresh vegetables. *Japan J Nutrit* 1990; **48:** 73-78.
23. Uribarri J, Woodruff S, Goodman S, *et al.* Advanced glycation end products in foods and a practical guide to their reduction in the diet. *J Amer Dietetic Assoc* 2010; **110:** 911-916.
24. Descombes E, Hanck AB, Fellay G. Water soluble vitamins in chronic hemodialysis patients and need for supplementation. *Kidney Internat* 1993; **43:** 1319-1328.
25. Kihm LP, Muller-Krebs S, Klein J, *et al.* Benfotiamine Protects against Peritoneal and Kidney Damage in Peritoneal Dialysis. *J Amer Soc Nephrol* 2011; **22:** 914-926.
26. Schupp N, Dette E, Schmid U, *et al.* Benfotiamine reduces genomic damage in peripheral lymphocytes of hemodialysis patients. *Naunyn-Schmiedeberg's Arch Pharmacol* 2008; **378:** 283-291.
27. Ariaey-Nejad MR, Pearson WN. Catabolism of 14C-Thiazole-labeled Oxythiamine and Its Effects on Thiamine Catabolism in the Rat. *J Nutrit* 1968; **96:** 206-214.
28. Rindi G, Patrini C, Nauti A, *et al.* Three thiamine analogues differently alter thiamine transport and metabolism in nervous tissue: An in vivo kinetic study using rats. *Metabolic Brain Disease* 2003; **18:** 245-263.
29. Casirola D, Ferrari G, Gastaldi G, *et al.* Transport of thiamine by brush-border membrane vesicles from rat small intestine. *J Physiol* 1988; **398:** 329-339.
30. Schwartz E, Manthei RW. Enhanced methylglyoxal toxicity in oxythiamine induced thiamine deficiency in mice. *Exp Biol Med* 1964; **116:** 1089-1091.
31. Rabbani N, Thornalley PJ. Dicarbonyls (Glyoxal, Methylglyoxal, and 3-Deoxyglucosone). *Uremic Toxins*. John Wiley & Sons, Inc., 2012, pp pp. 177-192.

32. Rindi G, de Giuseppe L, Ventura U. Distribution and Phosphorylation of Oxythiamine in Rat Tissues. *J Nutrit* 1963; **81:** 147-154.
33. Strumilo S, Czygier M, Markiewicz J. Different extent of inhibition of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase both containing endogenous thiamine pyrophosphate, by some anticoenzyme analogues. *J Enzyme Inhibition* 1995; **10:** 65-72.
34. Vanholder R, Dellaquila R, Jacobs V, *et al.* Depressed phagocytosis in hemodialyzed patients - invivo and invitro mechanisms. *Nephron* 1993; **63:** 409-415.
35. Yawata Y, Jacob H. Abnormal red cell metabolism in patients with chronic uremia: Nature of the defect and its persistence despite adequate hemodialysis. *Blood* 1975; **45:** 231-239.
36. Thornalley PJ. The potential role of thiamine (vitamin B1) in diabetic complications *Curr Diabetes Res* 2005; **1:** 287-298.
37. Bukhari FJ, Moradi H, Gollapudi P, *et al.* Effect of chronic kidney disease on the expression of thiamin and folic acid transporters. *Nephrol Dialysis Transplantat* 2010; **26:** 2137-2144.
38. Embden GD, H.J.; Kraft, G. Ober die intermediaren Vorgange bei der Glykolyse in der Muskulatur. *Klin Wochensch* 1932; **12:** 213-215.
39. Vuilleumier JP, Keller HE, Rettenmaier R, *et al.* Clinical chemical methods for the routine assessment of the vitamin status in human-populations. 2. The water-soluble vitamin-B1, vitamin-B2 and vitamin-B6. *Internat J Vitamin and Nutrit Res* 1983; **53:** 359-370.
40. Esakova OA, Meshalkina LE, Golbik R, *et al.* Which stage of the process of apotransketolase interaction with thiamine diphosphate is affected by the regulatory activity of the donor substrate? *IUBMB Life* 2007; **59:** 104-109.

41. Kopple JD, Dirige OV, Jacob M, *et al.* Transketolase activity in red blood-cells in chronic uremia. *Trans Amer Soc Artificial Internal Organs* 1972; **18:** 250-256.
42. Dreyfus PM. Clinical Application of Blood Transketolase Determinations. *New Engl J Med* 1962; **267:** 596-598.
43. Leunis JC, Vanriet C, Brauman J. 2-Step assay of erythrocyte transketolase activity. *Clinl Chem* 1982; **28:** 391-392.
44. Smeets EHJ, Muller H, Dewael J. NADH-Dependent transketolase assay in erythrocyte hemolysates. *Clin Chim Acta* 1971; **33:** 379-&.
45. Takeuchi T, Nishino K, Itokawa Y. Improved determination of transketolase activity in erythrocytes. *Clin Chem* 1984; **30:** 658-661.
46. Finglas PM. Thiamin. *Internat J Vitamin and Nutrit Res* 1993; **63:** 270-274.
47. Larkin JR, Zhang F, Godfrey L, *et al.* Glucose-Induced Down Regulation of Thiamine Transporters in the Kidney Proximal Tubular Epithelium Produces Thiamine Insufficiency in Diabetes. *PLoS ONE* 2012; **7:** e53175.
48. Brady JA, Rock CL, Horneffer MR. Thiamin status, diuretic medications, and the management of congestive heart failiure. *J Amer Diet Assoc* 1995; **95:** 541-544.
49. Steiber AL, Kopple JD. Vitamin Status and Needs for People with Stages 3-5 Chronic Kidney Disease. *J Renal Nutrit* 2011; **21:** 355-368.
50. Thore S, Frick C, Ban N. Structural Basis of Thiamine Pyrophosphate Analogues Binding to the Eukaryotic Riboswitch. *J Amer Chem Soc* 2008; **130:** 8116-8117.
51. Dawson RMC, Elliott DC, Elliott WH, *et al. Data For Biochemical Research*, vol. 3rd Oxford University Press: Oxford, 1989.
52. Chamberlain BR, Buttery JE, Pannall PR. A stable reagent mixture for the whole blood transketolase assay. *Ann Clin Biochem* 1996; **33:** 352-354.

53. Babaei-Jadidi R, Karachalias N, Ahmed N, *et al.* Prevention of incipient diabetic nephropathy by high dose thiamine and Benfotiamine. *Diabetes* 2003; **52:** 2110-2120.

54. Kochetov GA, Philippov PP, Razjivin AP, *et al.* Kinetics of reconstruction of holo-transketolase. *FEBS Letters* 1975; **53:** 211-212.

Table 1 | Characteristics of healthy subjects and ESRD patients on dialysis.

Variable	Healthy controls	CAPD	HD
Gender (M/F)	8/8	7/9	8/8
Age (years)	48 ± 5	43 ± 15	49 ± 6
BMI (kg/m^2)	25.5 ± 3.2	25.6 ± 3.0	26.2 ± 3.6
Plasma creatinine (μM)	71 ± 17	655 ± 249	677 ± 268
Plasma albumin (mg/ml)	45.6 ± 3.2	35.6 ± 6.9	43.1 ± 3.5
Plasma thiamine (nM)	5.1 (4.3- 9.7)	31.8 (18.0 – 53.5)***	51.1 (20.1 – 83.0)***
Plasma oxythiamine (nM)	0.18 (0.11 – 0.22)	0.64 (0.48 – 0.94)***	2.73 (1.52 – 5.76)***,^{OOO}

Data are mean ± SD or median (lower – upper quartile). Significance: *** and ^{OOO}, P<0.001 with respect to healthy controls and CAPD, respectively. A second study group of HD patients has plasma analysed for plasma oxythiamine before and after a dialysis session and characteristics were: gender (M/F) 6/6, age 49 ± 24 yrs, BMI 26.4 ± 7.9 kg/m^2, and plasma albumin 40.5 ± 4.0 mg/ml (n = 12).

Table 2 | Liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection of thiamine metabolites.

Analyte	Rt (min)		Molecular ion> fragment ion transition (Da)	Collision energy (eV)	Cone voltage (V)
	Method 1	Method 2			
Oxythiamine	12.9	36.5	265.9 > 123.0	18	40
Thiamine	13.9	39.5	264.9 > 122.0	18	16
4,5,4-Methyl-[$^{13}C_3$]-thiamine	13.9	39.5	267.9 > 122.0	18	16
OTPP	---	32.7	425.9 > 123.0	26	44
TPP	---	32.7	424.9 > 122.0	26	44

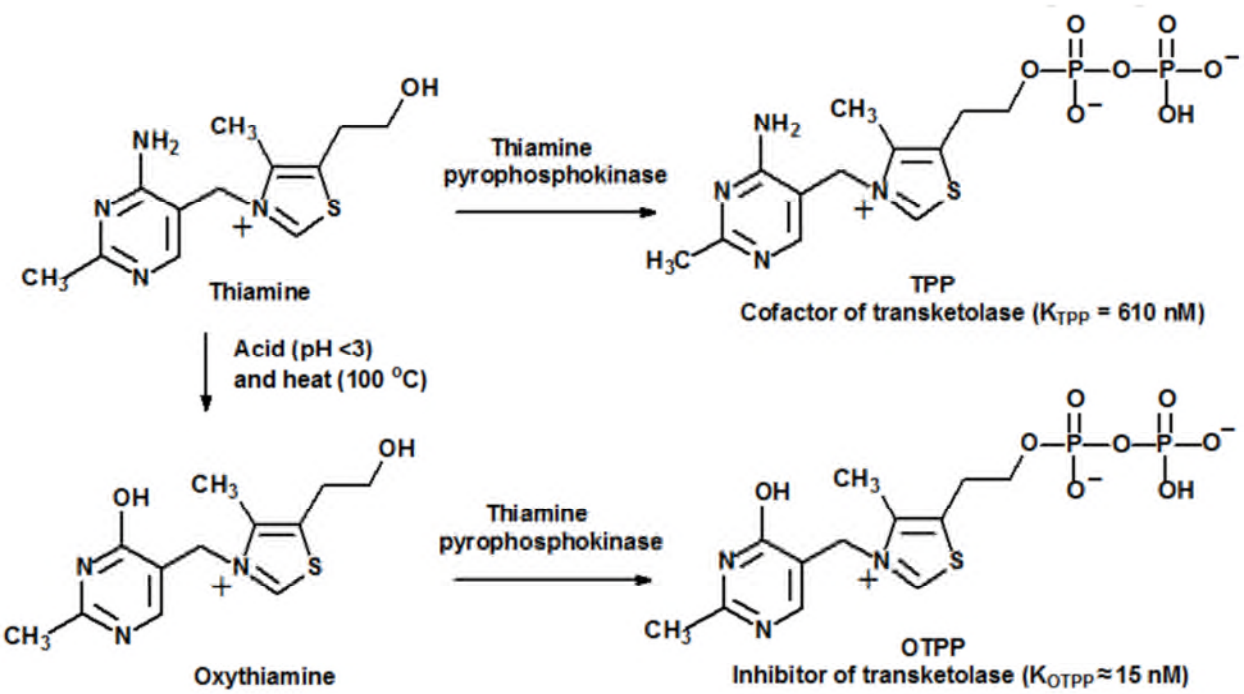
Chromatographic conditions: **Method 1.** Column - two graphitic Hypercarb™ columns (Thermo) in series, 5 µm particle size, 2.1 x 50 mm and 2.1 mm x 250 mm. The mobile phase was: 0.1% trifluoroacetic acid (TFA) with a custom gradient of acetonitrile (MeCN); 0.2 ml/min. Elution programme: 0 – 2 min, 0% MeCN; 2 – 15 min, 0 – 18% MeCN; and 15 – 18 min, 18 – 50% MeCN. After each injection the columns were cleaned by elution of column 1 and then column 2 with 50% tetrahydrofuran in 0.1% TFA for 5 min, 0.2 ml/min) and then re-equilibrated with 0.1% TFA in water, 0.4 ml/min, for 15 min. LC-MS/MS was performed with an Acquity-Quattro Premier (Waters) with electrospray source temperature 150 ^{o}C, desolvation gas temperature 500 ^{o}C with desolvation and cone gas flows of 1000 and 150 L/h respectively, and capillary voltage was 0.6 kV. **Method 2.** As for Method 1 except elution programme: 0 – 2 min, 0% MeCN; 2 – 45 min, 0 – 5% MeCN. LC-MS/MS was performed with an Acquity-Xevo-TQS (Waters) to meet the increased sensitivity of detection response required with instrument settings as above except capillary voltage was 2.5 kV.

FIGURE LEGENDS

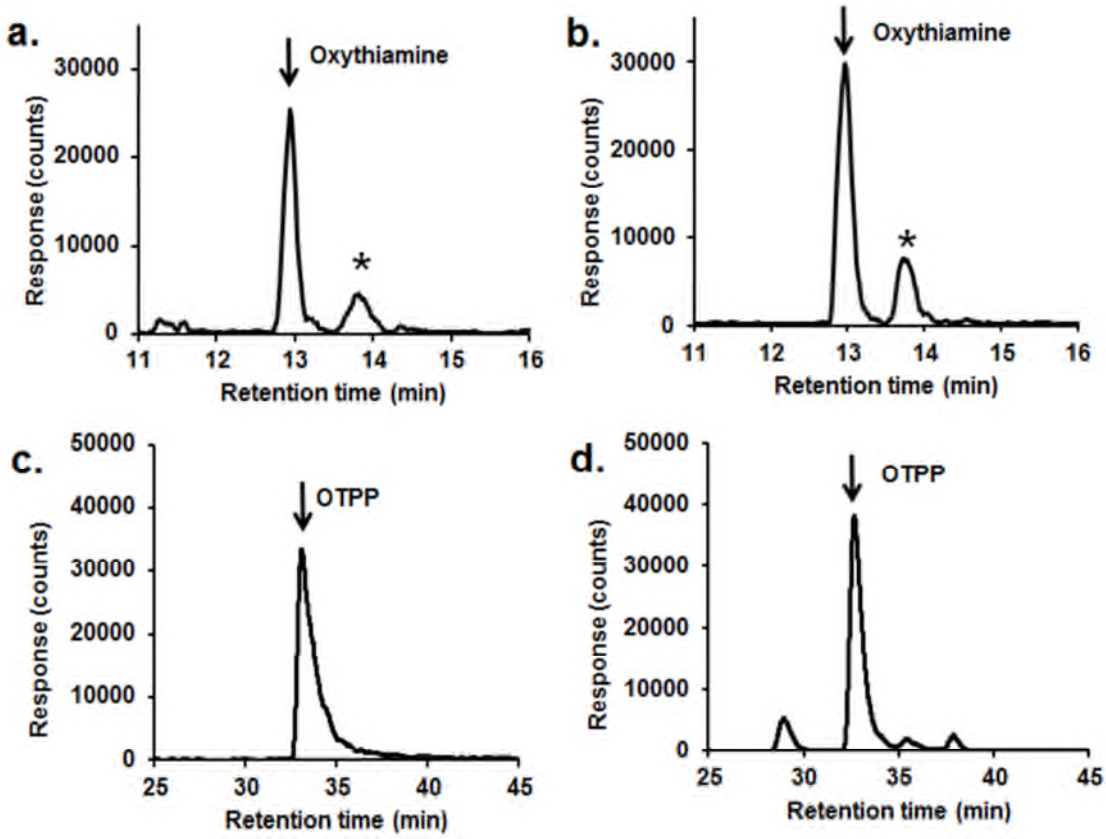
Figure 1 | Formation, metabolism and antimetabolite activity of oxythiamine. Pathways of thiamine metabolism, oxythiamine formation and metabolism to antimetabolite oxythiamine pyrophosphate.

Figure 2 | Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay of oxythiamine and oxythiamine pyrophosphate. Detection of oxythiamine, multiple reaction monitoring chromatogram, 265.9 > 123.0 Da: **a.** Analytical standard containing 100 fmol oxythiamine and 100 fmol thiamine. **b.** Detection of oxythiamine in plasma of a HD patient. * indicates oxythiamine formed from thiamine in the electrospray ionisation source. Detection of oxythiamine pyrophosphate (OTPP), multiple reaction monitoring chromatogram, 425.9 > 123.0 Da: **c.** Analytical standard containing 1.5 pmol fmol OTPP. **b.** Detection of OTPP in red blood cells of a HD patient.

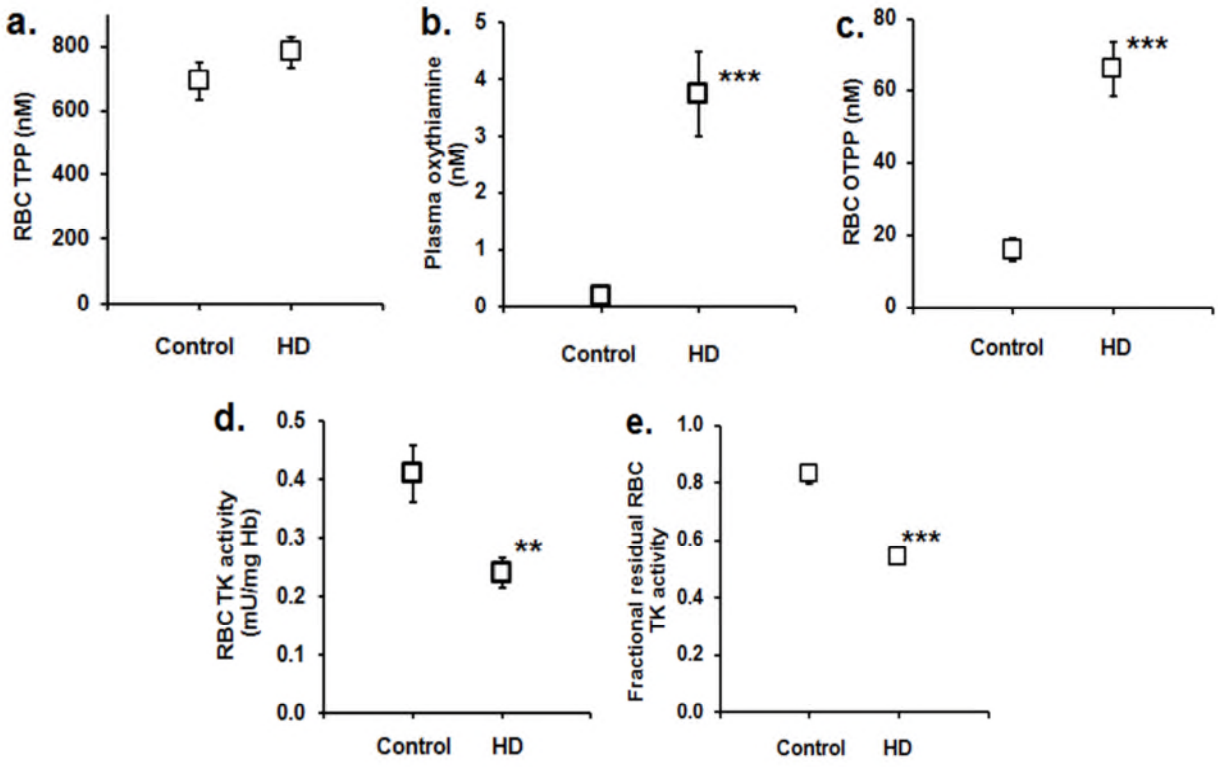
Figure 3 | Metabolism of oxythiamine to oxythiamine pyrophosphate (OTPP) and inhibition of red blood cell transketolase in healthy human subjects and HD patients. a. red blood cell (RBC) thiamine pyrophosphate (TPP), healthy controls, 692 ± 185 nM, and HD patients, 782 ± 176 nM (n = 13; P>0.05, *t*-test). **b.** Plasma oxythiamine concentration - Healthy controls, 0.19 ± 0.11 nM, and HD patients, 3.74 ± 2.97 nM (P<0.001, *t-test*). **c.** Red blood cell OTPP – Healthy controls, 15.9 ± 10.4 nM, and HD patients, 66.1 ± 26.7 (P<0.001, *t-test*). **d.** Red blood cell transketolase (TK) activity - Healthy subjects, 0.410 ± 0.144 mU/mg Hb, and HD patients, 0.240 ± 0.107 mU/mg Hb (- 41%, P<0.002, *t*-test). **e.** Fractional residual red blood cell transketolase activity – see Methods section. Data are mean ± SD (n = 10, healthy subjects n = 13, HD patients).



Zhang *et al.*, Endogenous thiamine antimetabolite etc., Fig. 1



Zhang *et al.*, Endogenous thiamine antimetabolite etc., Fig. 2



Zhang *et al.*, Endogenous thiamine antimetabolite etc., Fig. 3